Development of a high throughput *Pseudomonas aeruginosa* epithelial cell adhesion assay

Britta Swanson\textsuperscript{a, *}, Richard Savel\textsuperscript{a}, Frank Szoka\textsuperscript{b}, Teiji Sawa\textsuperscript{a}, Jeanine Wiener-Kronish\textsuperscript{a}

\textsuperscript{a}Department of Anesthesia and Perioperative Care and Cardiovascular Research Institute, San Francisco Medical Center, University of California, 513 Parnassus Avenue, S-261 San Francisco, CA 94127-0542 USA

\textsuperscript{b}Department of Biopharmaceutical Science, San Francisco Medical Center, University of California, 513 Parnassus Avenue, S-261 San Francisco, CA 94127-0542 USA

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Abstract

*Pseudomonas aeruginosa* colonizes the lungs of cystic fibrosis and mechanically ventilated patients by binding to specific carbohydrate residues on the surface of lung epithelial cells. Studies have shown that blocking this interaction may have therapeutic effects in vivo. To test compounds that may have an effect on the binding of *P. aeruginosa* to epithelial cells, we have developed a pseudomonal adhesion assay that is compatible with high throughput technology. This assay utilizes a 96-well culture plate assay and *P. aeruginosa* strains that have been modified to bioluminesce. This method has proven to be a rapid, sensitive and reproducible system for screening agents that inhibit bacterial adhesion.

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1. Introduction

*Pseudomonas aeruginosa* (PA) causes severe pulmonary infections in immunocompromised patients and in patients with cystic fibrosis (CF) (Hoiby, 1993; Hutchison and Govan, 1999). *P. aeruginosa* is the major culprit responsible for the morbidity and mortality seen in CF patients (Baltimore et al., 1989). Adhesion of PA to human airway epithelial cells is a key step in the establishment of respiratory colonization and infection (Barghouthi et al., 1996). Interestingly, *P. aeruginosa* does not avidly bind to normal, uninjured epithelial cell surfaces. In the presence of epithelial inflammation or injury (such as has been documented in CF or in mechanically ventilated patients) there is a dramatic increase in the ability of PA to bind (de Bentzmann et al., 1996). Therefore, blocking bacterial adherence might decrease lung pathology in patients at risk for *P. aeruginosa* infections.

The adherence of *P. aeruginosa* within the lungs has been shown to be mediated by type IV pili (Hahn, 1997) and flagella (Ramphal et al., 1996). The type IV pili, the major *P. aeruginosa* adhesin (Hahn, 1997), bind to a specific carbohydrate moiety of asialoGM, while flagella have been shown to adhere to carbohydrate moieties of Lewis x derivatives (Scharfman et
There is substantial data indicating that exogenous carbohydrates can reduce *P. aeruginosa* adherence to epithelial cells (Barghouthi et al., 1996; King et al., 2000; Plotkowski et al., 2001) and decrease adherence to collagen (Stepinska and Trafny, 1995; Trafny et al., 1995) by blocking receptors. Research has shown that the application of carbohydrates that block initial bacterial adhesin interactions may decrease lung infection in vivo (Ko et al., 1987; Mouricout et al., 1990). In one study of *P. aeruginosa* lung infection due to strain PAO1 instillation, 55% of control mice developed pneumonia, whereas only 13% of experimental mice pretreated with a dextran inhalant 2 h prior to *P. aeruginosa* exposure developed pneumonia (Bryan et al., 1999). Therefore, blocking adhesion of *P. aeruginosa* appears to have some clinical utility. To assess and compare the efficacy of multiple putative blocking agents, we developed an assay compatible with high throughput technology. This is a report of the development and reproducibility of this assay.

2. Materials and methods

2.1. Cell lines and *P. aeruginosa* strains

We utilized two different human tracheal epithelial cell lines for this study. BEAS-2B (ATCC #CRL9609, Rockville, MD) were maintained in RPMI medium supplemented with 5% heat-inactivated fetal calf serum (FCS) and 1 × penicillin/streptomycin (Pn/Sm). Calu-3 cells (Dr. Jonathon Widdicombe, UC Davis, CA) were maintained in 50% DME H-21/50% Ham’s F-12 medium supplemented with 5% heat-inactivated FCS and 1 × Pn/Sm. Both cell lines were grown at 37 °C with 5% CO₂ (Lee et al., 1999; Stark et al., 1992). For the adhesion assay, cells were grown in 96-well tissue culture-treated, opaque microtiter plate (Costar 3917) with the respective cell culture conditions until 90–100% confluent (approximately 2 × 10⁵ cells/well).

Several strains of *P. aeruginosa* were used in this study including PAK, PAKΔpilA, PAO1 and PA103. The PAK isogenic pilin mutant was a kind gift from Dr. S. Lory (Harvard University, USA). All strains were grown in LB containing 300 μg/ml carbenicillin (to maintain the luminescent plasmid) and the appropriate antibiotic to preserve the mutation (Tc, 60 μg/ml). Cultures were grown at 37 °C with aeration.

2.2. Construction of luminescent plasmid

A plasmid containing the luxCDABE operon was kindly donated by Dr. S. Swift (Winson et al., 1998). The lux operon was isolated using EcoRI and ligated into the pUCP19 vector (Schweizer, 1991), a derivative of pUC19 suitable for replication in *P. aeruginosa* (PA). Because the lac promoter of pUC19 is constitutively expressed in PA, no additional promoter for the lux operon was required. In addition, promoter activity was not significantly affected by the addition of carbohydrates used in this study. This plasmid (pBS110) was introduced into PA by electroporation as previously described (Enderle and Farwell, 1998).

2.3. Preparation of bacteria for adhesion

Bacteria were grown for 5–6 h to an optical density (OD₆₀₀) of approximately 2.0 (exponential growth). Five milliliters of each culture were pelleted by centrifugation at 9000 × g for 10 min at 4 °C, washed twice in 5 ml phosphate-buffered saline (PBS) and resuspended in 5 ml of PBS. Serial ten-fold dilutions of each bacterial strain were prepared (10⁻¹ to 10⁻⁷) in PBS and plated to verify colony-forming units (CFUs)/ml. PBS was used to reduce the level of light quenched by phenol red present in the cell culture medium. One hundred microliters of bacterial dilutions were applied to each well (1 × 10⁶ to 1 × 10⁷ bacteria. *Pseudomonas* strains carrying pBS110 were measured for luminescence using a 96-well plate luminometer (MicroLumat Plus, Perkin Elmer, Boston, MA).

2.4. Carbohydrates

We tested four standard carbohydrates used in adhesion assays (Sigma, St. Louis, MO). Three different concentrations of each carbohydrate (melezitose, melibiose, lactose and galactose) were prepared in PBS so that when 50 μl of each inhibitor was added to each well, the final concentration would be 25, 10 or 1 mM. If carbohydrates were not tested, PBS was added to the well.
3. Results

3.1. Creation of standard curves

To determine if the level of luminescence expressed by P. aeruginosa would be high enough for detection in the adhesion assay, we created standard curves for each bacterial strain. For each standard curve, we measured the quantity of bacteria (expressed as colony-forming units (CFUs/ml)) and the amount of luminescence expressed in relative light units (RLUs). First, serial dilutions were prepared for each strain (\(10^{-1}\) to \(10^{-7}\)). One hundred microliters of each dilution was measured for luminescence and the number of CFUs/ml in each strain was determined by colony plating. The logarithmic values of RLUs and CFUs/ml were then plotted to obtain a linear regression equation and a \(r^2\) value (coefficient of determination) for each bacterial strain: the standard curve (Fig. 1). Each strain showed a linear relationship between the number of bacteria and relative luminescence with a \(r^2\) value >0.95 and expression was the similar regardless of the strain of P. aeruginosa tested. In addition, luminescence could be detected from as few as \(1 \times 10^3\) bacteria.

3.2. Standard curve equation

Using the information obtained from the standard curve data, we created a linear equation to calculate the numbers of bacteria (CFUs/ml) in each experimental well based on the luminescence expressed. RLUs for each well were adjusted to subtract background measurements such that the negative controls (no bacteria) = 0 (adjusted RLUs). Plotting the standard curve, if \(y = mx + b\), where \(y\) and \(x\) are variables, \(m\) is the regression coefficient and \(b\) is the constant, then:

\[
\text{CFUs/ml} = 10^{(m(x)+b)},
\]

where

\[
x = \log(\text{adjusted RLUs}).
\]

3.3. Adhesion assay

Adherence of P. aeruginosa to human respiratory epithelial cells was quantified using a 96-well microtiter plate assay. Briefly, BEAS-2B or Calu-3 cells were grown to 90–100% confluence, the cell culture medium was then gently removed using a multichannel pipettor, and 50 \(\mu\)l of appropriate fresh cell culture media (without antibiotics) was applied. Bacteria were then added to each well with or without the addition of carbohydrates. Relative light units from each well were counted (5 s/well) immediately after addition of all components (media, bacteria and carbohydrates) and termed \(C_p\) for preincubation count. The plates were then incubated for 1 h at 37 °C to allow binding to occur. Plates were counted again (\(C_1\)) as the starting bacterial count before washing. The supernatant containing nonadherent bacteria was then removed using a multichannel pipettor and the adherent bacteria were washed three times with 200 \(\mu\)l PBS. A final volume of 200 \(\mu\)l (cell culture medium (50 \(\mu\)l) and PBS (150 \(\mu\)l)) was added to the wells and the plate was read again (\(C_2\)) in the luminometer. The

![Fig. 1. Standard curves of four different laboratory strains of P. aeruginosa expressing the luminescent plasmid pBS110.](image-url)
amount of bacteria that adhered to epithelial cells was expressed as

\[
\text{% adhesion} = \left( \frac{\text{C}_2 \text{ CFUs/ml}}{\text{C}_1 \text{ CFUs/ml}} \right) \times 100\%.
\]

Experimental conditions were repeated in triplicate and expressed as the average ± standard deviation (SD). A p-value < 0.05 based on Student’s t-test (InStat 2.01, GraphPad Software) was considered significant.

3.4. Optimization of assay conditions

We wanted to use the lowest multiplicity of infection (MOI) possible to reach numbers of bacteria that were physiologically relevant. However, since the relative numbers of bacteria that actually bind to epithelial cells is very low, we were concerned that these low numbers of bacteria would fall below the sensitivity of the luminometer (\( \sim 1 \times 10^3 \text{ CFUs/ml} \)). As a negative control for Pseudomonas adherence, isogenic pilin mutants of each strain were tested and compared to wild-type strains. Using as many as \( 1 \times 10^9 \text{ CFUs/well} \) (MOI = 10,000:1) or as few as \( 1 \times 10^6 \text{ CFUs/well} \) (MOI = 10:1), a significant difference in adhesion to the epithelial cells was observed when the pilin mutants were compared to the wild-type parent strain (data not shown). Even using an MOI of 10:1, we were able to detect light units from the pilin mutant in which only 1–2% of bacteria adhered, representing approximately \( 2 \times 10^4 \text{ CFUs/ml} \) (Fig. 2A and B, lanes 1 and 2). This number was verified by manually plating the bound bacteria.

![Figure 2](image_url)

Fig. 2. Adhesion of P. aeruginosa PAK to epithelial cells in the presence of 1, 10 or 25 mM carbohydrates. (A) BEAS-2B epithelial cells. (B) Calu-3 epithelial cells. No carbohydrate = PAK alone; PAKpilA, PAK pilin mutant; Lac, lactose; MB, melibiose; MZ, melezitose; Gal, galactose. Bars indicate the average ± standard deviation of three independent wells.
3.5. Inhibition of bacterial adhesion by carbohydrates

Four different carbohydrates were initially tested for their ability to block *P. aeruginosa* adherence to respiratory epithelial cells: lactose, melibiose, melezitose and galactose. Carbohydrates were incubated with the bacteria 10 min before application to the epithelial cells, or were incubated with the cell culture before application of bacteria. There was no significant difference in bacterial adhesion between the two methods of carbohydrate application (data not shown). In both cell lines tested, a significant difference in binding activity was observed between wild-type PAK and its isogenic pilin mutant. Notable differences were also observed when PAK was incubated with epithelial cells in the presence of 25 mM of any sugar, 10 mM melibiose or 10 mM melezitose.

4. Discussion

Many different types of adhesion assays have been utilized in the past. The greatest limiting factor in these assays has been determining the best way to quantitate bacterial adhesion. Simple adhesion assays using unaltered bacteria require bacterial plate counting or electron microscopy. Both methods are labor-intensive and time-consuming. More advanced approaches utilize tagged bacteria such that they can be detected without the use of plating, including fluorescence labels and radioactivity (Falk et al., 1994; Plotkowski et al., 1991; Scharfman et al., 1999). Although these techniques provide results more rapidly, they suffer from the requirement for an exogenous light source, the long half-life of fluorescent protein or the problems inherent in utilizing radioactive labels. We have developed an assay which utilizes bioluminescence produced from the *luxCDABE* operon of *Photorhabdus luminescens* (Winson et al., 1998) to detect the bacteria, a less unwieldy technique that provides an accurate measure of the presence of metabolically active bacteria without any of the drawbacks mentioned above. This system is superior to the green fluorescent protein technique and other luminescent systems of bacterial detection because: (1) both substrate and enzyme are produced endogenously so that no exogenous sub-

strate must be added; (2) the half-life of the luminescence is significantly less than that of gfp, allowing real-time observation of metabolically active bacteria; (3) luminescence does not require excitation by an exogenous light source; (4) bioluminescence excludes the need for radioactivity. The *luxCDABE* operon has been used successfully in several strains of bacteria for detection, including *P. aeruginosa* for studying biofilm susceptibility to antimicrobials (Parveen et al., 2001). In addition, bioluminescence is proving to be an accurate indicator of true numbers of bacteria (Stollenwerk et al., 1998; Uesugi et al., 2001).

This system has many applications for screening large numbers of potentially therapeutic agents for a variety of experiments. In addition, luminescent bacteria can be used in combination with cell culture for adhesion studies as described above such that the focus can be on the cell type used or on the bacteria. For example, the difference in relative adhesion of *P. aeruginosa* to the two cell lines utilized in our studies was notable. This difference can be attributed to alternative cell surface structures and/or differences in the growth of the monolayer. Calu-3 cells normally produce confluent monolayers with tight junctions, but have been shown to bind *P. aeruginosa* more efficiently when there are “free edges” due to alterations in monolayer integrity (Lee et al., 1999). Alternatively, testing bacteria at different stages of growth may affect their ability to adhere due to differential expression of adhesion factors. This assay could also be utilized in combination with gentamycin-protection assays in which viable, intracellular bacteria are quickly and easily detected. We are currently using the assay to screen several synthesized polysaccharide compounds for their ability to inhibit *P. aeruginosa* adhesion.

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References


